Original: Japanese
Provisional translation

Analytical Method for Spiromesifen (Animal and Fishery Products)

1. Analytes

Spiromesifen

4-Hydroxy-3-mesityl-1-oxaspiro[4.4]non-3-en-2-one (hereinafter referred to as metabolite M1)

4-Hydroxy-3-(4-hydroxymethyl-2,6-dimethylphenyl)-1-oxaspiro[4.4]non-3-en-2-one (hereinafter referred to as metabolite M2, including its conjugates.)

2. Application

Animal and fishery products

3. Instrument

Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)

4. Reagents

Use the reagents listed in Section 3 of the General Rules, except the following.

Reference standard of spiromesifen: Contains not less than 98% of spiromesifen.

Reference standard of metabolite M1: Contains not less than 98% of metabolite M1.

Reference standard of metabolite M2: Contains not less than 98% of metabolite M2.

5. Procedure

1) Extraction

Weigh the sample accurately, add a mixed solution of ethanol, formic acid and water (9:2:9) in a volume of 3/10 by weight, homogenize, and take the sample equivalent to 10.0 g (5.00 g for fat). Add 100 mL of acetonitrile, formic acid and water (80:1:20) mixed solution and 20 mL of *n*-hexane, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the mixed layer of acetonitrile and water. Add 50 mL of acetonitrile, formic acid and water (80:1:20) mixed solution to the *n*-hexane layer and the residue, homogenize, centrifuge as described above, combine with the resulting mixed layer of acetonitrile and water and add a mixed solution of acetonitrile, formic acid and water (80:1:20) to make exactly 200 mL. Take exactly a 5 mL (10 mL for fat) aliquot of the solution and concentrate to approximately 1 mL at below 40°C. Add 0.1 mL of 0.1 vol% formic acid and 40 mL of 10 w/v% sodium chloride solution and extract with shaking twice with 50 mL each of ethyl acetate and *n*-hexane (1:19) mixed solution. Combine the extracts and consider them the fraction of spiromesifen and metabolite M1 (Fraction I). Collect the remaining aqueous layer and consider it the fraction of metabolite M2 and metabolite M2 conjugate (Fraction II). Dehydrate Fraction I with anhydrous sodium sulfate, filter out anhydrous sodium sulfate, concentrate at below 40°C, and remove the solvent. Dissolve the residue in a mixed solution of acetonitrile and 0.01 vol% formic acid (1:1) to make exactly 10 mL and use this solution

as the test solution for spiromesifen and metabolite M1.

2) Hydrolysis (only for animal products)

Add water to Fraction II obtained in 1) to make exactly 50 mL. Take exactly a 10 mL aliquot of the solution, add 6 mL of hydrochloric acid, seal tightly and heat in an oil bath at 90°C for 3 hrs to hydrolyze the metabolite M2 conjugate. Allow to cool, add 100 mL of 10 w/v% sodium chloride solution, and extract twice with shaking with 100 mL and 50 mL each of ethyl acetate and *n*-hexane (1:1) mixed solution. Combine the extracts, dehydrate with anhydrous sodium sulfate, filter out anhydrous sodium sulfate, concentrate at below 40°C, and remove the solvent. Dissolve the residue in 10 mL of acetonitrile and acetic acid (99:1) mixed solution.

3) Clean-up

Inject 10 mL of acetonitrile and acetic acid (99:1) mixed solution into an ethylenediamine-*N*-propylsilanized silica gel cartridge (500 mg), and discard the effluent. Transfer the solution obtained in 2) to the cartridge and discard the effluent. Then, add 20 mL of acetonitrile and acetic acid (19:1), concentrate the eluate at below 40°C, and remove the solvent. Dissolve the residue in a mixed solution of acetonitrile and 0.1 vol% formic acid (1:1) mixed solution to make exactly 2 mL, and use this solution as the test solution for metabolite M2.

6. Calibration curve

Dissolve each reference standard of spiromesifen, metabolite M1 and metabolite M2 in acetonitrile respectively to make standard stock solutions. Mix each stock standard solution appropriately, dilute with a mixed solution of acetonitrile and 0.1 vol% formic acid (1:1) to prepare standard solutions of several concentrations. Inject each solution into LC-MS/MS, and make calibration curves by peak-height or peak-area method. When the test solution is prepared following the above procedure, the concentration of each analyte in the test solution corresponding to 0.01 mg/kg (equivalent to spiromesifen) in the sample is 0.00025 mg/L (equivalent to spiromesifen).

7. Quantification

Inject the test solution into LC-MS/MS and calculate each concentration of spiromesifen, metabolite M1 and metabolite M2 from the calibration curve prepared in **6**. The concentration of spiromesifen including metabolite M1 and metabolite M2 is calculated using the following equation.

Concentration of spiromesifen (including metabolite M1 and metabolite M2) (ppm) = A + B × 1.360 + C × 1.285

A: Concentration (ppm) of spiromesifen

B: Concentration (ppm) of metabolite M1

C: Concentration (ppm) of metabolite M2

8. Confirmation

Confirm using LC-MS/MS.

9. Measurement conditions

(Example)

Column: Octadecylsilanized silica gel, 2.1 mm inside diameter, 150 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from a mixed solution of acetonitrile and 0.1 vol% formic acid (1:4) to (9:1) in 10 min and hold at (9:1) for 6 min.

Ionization mode: ESI (+)

Major monitoring ions (*m/z*)

Spiromesifen: Precursor ion 273, product ions 255, 187

Metabolite M1: Precursor ion 273, product ions 255, 187

Metabolite M2: Precursor ion 289, product ions 271, 241

Injection volume: 10 μL

Expected retention time

Spiromesifen: 15 min

Metabolite M1: 11 min

Metabolite M2: 9 min

10. Limit of quantification

0.01 mg/kg for each analyte (equivalent to spiromesifen)

11. Explanatory note

1) Outline of analytical method

The method consists of extracting spiromesifen, metabolite M1 and metabolite M2 (including its conjugates) from sample homogenized with a mixed solution of ethanol, formic acid and water (9:2:9) using a mixed solution of acetonitrile, formic acid and water (80:1:20) in the presence of *n*-hexane, fractionating spiromesifen and metabolite M1 into the organic layer and metabolite M2 and its conjugates into the aqueous layer by liquid-liquid partition using a mixed solution of ethyl acetate and *n*-hexane (1:19), for the organic layer, leaving as it is or for the aqueous layer, hydrolyzing the conjugates of metabolite M2 with hydrochloric acid to metabolite M2 followed by extracting with a mixed solution of ethyl acetate and *n*-hexane (1:1), then, cleaning up with an ethylenediamine-*N*-propylsilanized silica gel cartridge, and quantifying and confirming using LC-MS/MS. In this method, spiromesifen, metabolite M1 and metabolite M2 (including its conjugates) are quantified respectively. For the concentration of spiromesifen including metabolite M1 and metabolite M2 (including its conjugates), metabolite M1 and metabolite M2 are converted to the concentration of spiromesifen by multiplying by the conversion factor and the sum of the concentration of spiromesifen, metabolite M1 and metabolite M2 is regarded as the analytical result of spiromesifen. Pay attention to the analytes in each food when calculating the analytical result.

2) Notes

i) To prevent spiromesifene from decomposing during preparation and testing, a mixed solution of ethanol, formic acid and water must be added to the preparation and subsequent testing must also be performed under acidic conditions using formic acid.

ii) When the analytical method for spiromesifen, metabolite M1 and metabolite M2 using LC-MS/MS was developed, the following monitoring ions were used:

Spiromesifen

for quantitative ions (*m/z*): precursor ion 273, product ion 187

for qualitative ions (*m/z*): precursor ion 273, product ion 255

Metabolite M1

for quantitative ions (*m/z*): precursor ion 273, product ion 187

for qualitative ions (*m/z*): precursor ion 273, product ion 255

Metabolite M2

for quantitative ions (*m/z*): precursor ion 289, product ion 241

for qualitative ions (*m/z*): precursor ion 289, product ion 271

iii) Food items used to develop the analytical method: cattle (muscle, fat, liver and milk), chicken (muscle and egg), honey, eel, salmon and *Corbicula* (freshwater clam).

12. Reference

None

13. Type

C